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THE EFFECT OF BIOLOGICALLY CONDITIONED WATER UPON RATE OF GROWTH IN FISHES AND AMPHIBIA

A DISSERTATION SUBMITTED TO THE
GRADUATE FACULTY IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

By
GRETCHEN SHAW

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THE EFFECT OF BIOLOGICALLY CONDITIONED WATER UPON RATE OF GROWTH IN FISHES AND AMPHIBIA ¹

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INTRODUCTION

The rate of growth of an animal offers an excellent index for the determination of the effect of certain experimental conditions. In his book on *Animal Aggregations*, Allee ('31) has devoted two chapters to the problem of the effects of crowding on growth, in which he covers very completely the literature dealing with both the beneficial and harmful effects. It may be well to review here briefly papers dealing with fish and amphibia.

Bilski ('21) reported experiments with different numbers of *Bufo* larvae in like-sized aquaria, and with the same number of larvae in different sized vessels. With decreasing volume per animal, he found that there were descending averages of weight increase. The same author in a 1926 paper concluded that there was a greater regeneration of amputated tails of *Rana esculenta* with decreased volume per animal.

Goetsch ('24), experimenting on *Hydra*, *Planaria*, and amphibian larvae, found that while with the two less active forms, food or the concentration of excretory products played the most important rôle, with the amphibia, if food was controlled, the retarding factor seemed to be overstimulation due to confinement in a small space.

Church ('27) and Shaw ('29), in investigating the effect of homotypic conditioning on the rate of growth in fishes, found that when the water was unchanged over periods of ten days or more, the greatest growth occurred in tanks having the smallest number of fish, although there were exceptions to this, particularly in the early stages of long continued experiments.

Kawajiri ('28), studied the influence of population density of fishes on survival rate and rate of growth. He concluded that, for rainbow trout, the survival rate increased and the rate of growth decreased as the number of fry in a box increased.

Adolph ('29) found that with amphibian larvae the maximum growth occurred with a single animal in the largest volume, although conditioned water could favor the growth of the individual. He found less negative effect of crowding in youth. In his 1931 paper, he reported that the deficiency of body weight of the tadpoles, effected by crowding, retarded metamorphosis.

¹ The present investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation administered by W. C. Allee.

Carpenter ('30), found that lead nitrate, by reaction with the mucus secreted by the epithelial cells, made a film over the gills of fish which caused death by suffocation. Survival time in a given concentration was directly influenced by the changes in the ratio of volume of solution to mass of animal. Also, survival time was increased for later members of a series allowed to die successively in a given solution, because of precipitation of the metal by earlier members of the series.

Allee and Bowen ('32) in experiments on the protective effects of groups of goldfish exposed to colloidal silver, determined that there was definite group protection, due to the fact that a greater amount of the colloid was precipitated by the greater number of fishes. The amount of silver removed from suspension was found to correspond exactly with that recovered from the precipitate formed by the mucous secretions.

While the majority of the evidence favors the conclusion that homotypic crowding has a deleterious effect on growth, there are certain exceptions which suggest that this may not always be the case. These possibilities were explored in the present work. Certain experiments on heterotypic conditioning were also undertaken.

EXPERIMENTATION

The experiments to be reported fall into three general divisions. The first division covers experiments dealing with the effect of the conditioning of the medium by animals of a different species (heterotypic conditioning) on growth and regeneration. The second covers experiments on the effect of the conditioning of the medium by animals of the same species (homotypic conditioning) on growth and regeneration. The third is on the effect of heterotypic conditioning on the growth of animals subjected to sublethal doses of toxic substances. The last was suggested by certain experiments of Shelford ('17, '18) on stream pollution, in which he found that fish did not show a negative reaction to water containing certain toxic substances; the experiments in division three were planned with a view to discovering the effect of the presence of associated animals.

Fishes and amphibian larvae were used as experimental animals. The fish were of the tropical species *Platylocilus maculatus* and *Girardinus guppyi*. The amphibia were *Ambystoma*, of two species, *tigrinum* and *maculatum*. The age of the fishes used ranged from one to four or five months. They were hatched in the Whitman vivarium, and kept in stock aquaria, containing sand and plants, until ready for use. They were fed daily during the period of experimentation. The egg masses of *Ambystoma* were collected in the field and brought into the laboratory. The masses were then isolated, so that experimental and control animals could be taken from the same mass. The larvae were used as soon as possible after hatching, except in one case where it was desired to test animals which had been in the laboratory for some weeks. In

all but this one case the tadpoles were subsisting on yolk and hence needed feeding neither before nor during the experiments.

The water used throughout was from the well in the Whitman vivarium. The vessels in which the animals were kept for experimentation were lined with paraffin in every set of experiments except the first one reported. This was done to prevent the growth of algae, and to make possible the replacement of entirely clean linings at desired intervals.

All experiments on fishes were run in the Whitman vivarium, which has an annual temperature range of 19° – 26° C., but is usually at 22° C. The tadpoles were kept in the laboratory proper at a room temperature of 22° C.

I. HETEROTYPIC CONDITIONING

To test the effect of the presence of an associated animal on the fish and amphibia, fresh water mussels of the genera *Anodonta* and *Lampsilis* were used. It was not feasible to have the experimental animals directly associated with the mussels, as the slime they produce has definite food value. The procedure, therefore, was as follows: the mussels were isolated in a large container and the water, which had been conditioned for twenty-four hours, was siphoned from this container through filter paper, to remove the slime, then into the vessels containing the experimental animals. The water for the controls stood in a vessel beside that containing the mussels, so that, as they were both allowed to stand an equal length of time before use, there was sufficient opportunity for both to reach equilibrium. In certain experiments, as explained below, the water was aerated. The control water was also filtered in all except the first two sets of experiments reported.

A. Growth

a. *Platypoecilus*

In these experiments assembled aquaria with glass bottoms and sides, holding eight liters of water, were used. Two fishes were put in each experimental and two in each control aquarium. In this set the water for the controls was not filtered. The fishes were fed ten milligrams of prepared fish food daily. The food was left in for half an hour; at the end of this time all the food which had not been eaten was removed by siphoning. While removing food traces four liters of water were siphoned off, and the aquaria were refilled immediately from the stock containers of conditioned or control water as the case might be. Weekly the aquaria were cleaned and the water was changed entirely. The fishes were measured at the beginning of the experiment and at the end of thirty days. They were measured by putting them in a miniature aquarium whose sides were microscope slides, five millimeters apart, with a millimeter rule fastened to one side. Thus confined, the fishes soon became quiet, and measurements were read with a hand lens, with an experimental error of 0.1 mm. Weighing was not possible since the handling necessary to such a process kills these fish.

The results of these experiments will be found in table I (series I). At the end of the thirty days the average percentage increase was 6.3 per cent for those in conditioned water, and 2.7 per cent for those in unconditioned water. The twenty-one cases give a statistical significance by Student's method of .0044.

These results indicated strongly that fish would grow more rapidly in water which had been conditioned in the manner described. In order to test for any possible food value remaining even in filtered mussel-conditioned water, experiments were run just as has been described except that the fish were given no food (series II). The paraffin linings were replaced at the end of fifteen days, in addition to the washing at the end of seven days. If growth were to occur in the experimental tanks, there would be ground for suspicion that the food factor had not been ruled out.

Practically no growth occurred in the twenty cases (*i.e.*, in only four of the forty tanks—three experimental, one control). From an average size at the beginning of 18.1 mm. for the fish in conditioned water and 18.2 mm. for the controls, there was an average decrease in length of 0.7 per cent and 1.9 per cent respectively. This difference is not significant. There did, however, seem to be some slight survival value of the conditioned water, as the experimental fish lived an average of 28 days, and the controls an average of 25 days. This difference is not significant (.7762).

b. *Ambystoma*

The vessels used for the experiments on tadpoles were finger bowls, holding 200 cc. of water, and lined with paraffin. One animal was placed in each bowl. The water used for the experimental animals was aerated while being mussel-conditioned, and was then filtered. The aeration was introduced to improve the condition of the mussels, and was accomplished by bubbling a slow stream of air through the water continuously. The water for the controls was also aerated and filtered, in this and all subsequent experiments involving mussels. These experiments ran for seven days, and the water was unchanged during that time.

As it was desired to have growth experiments comparable with those on tail regeneration, the tail only was measured, after having ascertained that the tail was a reasonably constant fraction of the total length. The measurement was done under the low power of a binocular dissecting microscope, with the animals resting on a thin piece of glass under which was a millimeter rule. The animals remained quiet and the length was measured with certainty to tenths of a millimeter.

The results, seen in table I (series III), show a beneficial effect of conditioned water. In twelve out of fifteen cases the tadpoles in the conditioned water showed the greater increase in tail length; in two cases there was no difference.

B. Regeneration

In all regeneration experiments, the length of the tail was measured first, then approximately the desired amount was amputated; the length of the remaining tail stump was then measured. At the end of the time of experimentation, the total length of the tail was again measured. Thus by subtracting from this the length of the tail stump, the amount of increase was determined.

a. *Platypoecilus*

The tails of the fishes were removed with fine sharp scissors, while they were in the miniature aquarium. This obviated the bad effects, discovered in preliminary experiments, caused by removal from water for cutting. The amount remaining after cutting varied from 0.2 mm. to 1.0 mm. with an accuracy similar to that in all measurements of fishes.

For these and all subsequent experiments on fish, battery jars were used which had a circular bottom six inches in diameter and a height of eight inches. They were lined with paraffin, which was not renewed in the course

TABLE I. Summary of data on series I to V inclusive

Series no.	Species used	No. of exps.	Duration	Heterotypic conditioning						Statistical significance
				Conditioned water			Control			
				Mean length at start	Mean length of tail stump	Mean per cent inc.	Mean length at start	Mean length of tail stump	Mean per cent inc.	
I	<i>P. rubra</i>	21	30 da.	12.5*		6.3	12.5		2.7	.0044
II	<i>P. rubra</i> (starved)	20	30 da.	18.1*		-0.7	18.2		-1.9	.1054
III	<i>A. tig.</i>	15	7 da.	5.5		28.9	5.5		25.9	.0254
IV	<i>P. rubra</i>	13	14 da.	3.6	0.5	51.0	3.7	0.5	40.5	.0076
V	<i>A. mac.</i>	19	7 da.	4.8	1.0	48.7	4.8	1.1	42.7	.0006

* Total length (all others tail length).

Expanded tables have been placed in the University of Chicago library. The type of data available can be ascertained from table III, series XIV, which is given in full.

of these experiments. The experiments ran two weeks. The jars were filled to 1700 cc., which was about half full. Two fishes were put in each jar; they were chosen so as to be distinguishable either by size or markings. They were fed prepared fish food daily. An amount in excess of that which they could consume in half an hour was given them. At the end of that time they were removed from the jars with a small net, and put in a dish of clean water. Separate nets were used for experimental and for control animals. The jars were emptied, rinsed repeatedly to remove all traces of food, then filled with aerated water filtered from the stock containers. The fishes were replaced by pouring.

Of the thirteen cases (with twenty-six fishes) twelve showed increased growth in the conditioned water, giving a statistical significance of .0076 (table I, series IV).

b. *Ambystoma*

In regeneration experiments on tadpoles, the tail amputation was done with a sharp knife-needle, under the binocular dissecting microscope. Removal from the water for the few moments necessary seemed to have no ill effects. The length of the tail stumps varied from 0.6 mm. to 1.5 mm., in experiments in heterotypic conditioning.

Of the nineteen experiments of this type on thirty-eight tadpoles, fifteen were positive, two were negative, and two indifferent, showing a great preponderance of evidence for greater growth in the conditioned water. The figure for significance is .0006. The results will be found summarized in table I, series V.

It has been shown, then, that heterotypic conditioning has a beneficial effect on the rate of growth and the rate of tail regeneration in the fish *Platypoecilus*, and the amphibian *Ambystoma*.

II. HOMOTYPIC CONDITIONING

A. Growth

No work was done on fish in this connection, as this aspect is being covered by other investigations in this laboratory.

Ambystoma

To test the effect of homotypic conditioning, five tadpoles were placed in one of the paraffin-lined finger bowls holding 200 cc. of water. For control, five others were isolated in five similarly treated bowls. The experiments ran seven days with no change of water.

1. In the first set, the water was taken directly from the well-water tap, filtered, and put into the bowls. In examining table II, series VI, we find that there was not much difference manifested between the thirty isolated animals and the thirty animals living in six groups. The figure for significance is .1940, which shows only a slight tendency toward a positive difference. Each pertinent figure in the table is an average for five animals.

2. It was desired to test the responses of animals which had lived several weeks in the laboratory. Such animals were placed, therefore, in experimental conditions similar to those described above; the water was filtered and put into the bowls without being allowed to stand for any period of time before starting the experiment. The results produced under these conditions are summarized in table II, series VII. Each figure is again an average for five animals. There was a decrease in tail length in all but one case, but the decrease was less in the isolated animals in all cases. The significance of the difference is .0230; that is, there is a definite negative effect of crowding on the rate of growth of animals which have been in the laboratory for a considerable period.

3. Following the suggestion from the work of Bilski and Goetsch that the cause of negative effects of crowding was the stimulation produced by tadpoles colliding with each other, or with the sides of the vessels in small volumes, the following experiments were set up: the bowl to contain the grouped animals was divided into five equal parts by netting which permitted the free circulation of water. In the bowls for the controls the netting was so arranged as to set off a space exactly similar to one of the five divisions of the experimental bowl. Both vessels were filled with the same amount of the usual filtered well-water. By this means it was assured that each of the five experimental animals was confined to the same space as each one of the controls, and that the amount of medium per animal was five times as great for the latter. The arrangement also prevented collisions between animals. The thirty animals used showed almost no difference in growth. The difference shown (table II, series VIII) is not significant.

4. It was thought possible that a protective effect of crowding might be manifested with well water taken directly from the tap and not filtered, as there might be slightly harmful substances suspended in this which were removed by filtering. In this set of experiments only two cases of the twelve were indifferent; the rest were positive, giving a significance of .0002. Each pertinent figure (table II, series IX) is an average for five animals. These experiments are of especial interest, as they very strongly indicate that under certain conditions crowding has a beneficial effect on growth in tadpoles.

B. Regeneration

a. *Platypoecilus*

In these experiments the effect of conditioning was tested by the use of conditioned water instead of by crowding, since the conditioned water method had proved more effective in certain experiments on growth in fishes. A large stock aquarium, lined with paraffin, was filled with thirty-four liters of water, and about thirty fishes (*Platypoecilus*) were put in it. Water for the controls was in another paraffin lined container. Both control and conditioned water containers stood a day before the experiment was begun. The water was then siphoned from the stock containers into paraffin lined battery jars, which contained the fishes used for the experiment. The battery jars were filled to 1700 cc., and the two fishes placed in each. The procedure for feeding and change of water was similar to that described for the experiments on the effect of heterotypic conditioning. The amount of water taken from the stock experimental tank daily, for the renewal of water in the jars, was about half the amount present; the stock fishes were fed and the water completely renewed at the end of a week. Aeration was not necessary in order to keep the fishes in good condition (as had been the case with the mussels, which are stream forms) and the water was not filtered.

TABLE II. Summary of data on series VI to XIII, inclusive

Series no.	Species used	No. of expts.	Duration	Homotypic conditioning						Statistical significance	Remarks
				Isolated			Grouped				
				Mean tail length at start	Mean length of tail stump	Mean per cent inc.	Mean tail length at start	Mean length of tail stump	Mean per cent inc.		
VI	<i>A. tig.</i>	6	7 da.	5.1		31.3	5.1		34.4	.1940	Young larvae, filtered water
VII	<i>A. mac.</i>	8	7 da.	6.9		-4.6	6.9		-11.5	.0230	Older larvae, filtered water
VIII	<i>A. tig.</i>	15	7 da.	4.8		38.2	4.8		40.6	.4370	Young larvae, filtered water, in frames
IX	<i>A. tig.</i>	12	7 da.	5.7			5.6		10.1	.0002	Young larvae, untreated water
X	<i>P. rub.</i>	11	14 da.	5.0	0.8	44.0	5.0	0.9	36.0	.0158	
XI	<i>A. mac.</i>	10	7 da.	6.9	1.0	28.2	6.9	1.0	35.0	.0008	Untreated water
XII	<i>A. mac.</i>	10	7 da.	6.3	1.0	34.6	6.3	1.0	38.7	.0128	Filtered aerated water
XIII	<i>A. tig.</i>	7	7 da.	5.9	0.9	24.7	5.9	0.9	28.8	.0010	Untreated water

The experiments of series X, involving twenty-two fishes, ran two weeks. The length of the tail stumps varied from 0.3 mm. to 1.1 mm. The measurements at the end showed that all the fishes in conditioned water had grown more than their controls (see table II). The figure for significance is .0158, which indicates that homotypic conditioning has a positive effect on regeneration.

b. *Ambystoma*

In these experiments five animals were grouped in one finger bowl and five isolated in five bowls, as has been previously described in experiments where no frames were used.

It had been necessary to use both *A. tigrinum* and *A. maculatum* in the experiments on the effect of homotypic conditioning on growth. It was thought that the species were sufficiently closely allied so that they would give comparable reactions to experimental conditions. When the first experiments on regeneration were completed, in which *A. maculatum* had been used, it was found that there were enough fresh *tigrinum* on hand to repeat what had been done. This comparison would offer a good test for the above assumption.

No further detailed description is needed of the method employed in these experiments. The manner of tail amputation has been described in a previous section, and the animals were grouped as stated above. The length of the tail stumps varied from 0.8 mm. to 1.2 mm.

The first set (*A. maculatum*, series XI) was in well-water taken from the tap without aeration or filtration. Nine of the ten groups of five showed a higher rate of regeneration than their controls. The significance is .0008.

The second set (series XII) was in filtered and aerated water. A similar result occurred here, giving a significance of .0128. Considering the two sets together, there is almost no statistical chance that the results lack significance.

The conditions for the *tigrinum* experiments (series XIII) were a repetition of those for the first set of *maculatum* experiments, in untreated well-water. All the grouped animals showed a greater increase in growth than the controls. The statistical significance is .0010. These results are in close agreement with those given above for *maculatum*, which offers support for the assumption that the two species react similarly to these experimental conditions.

The results of these three sets of experiments are summarized in table II under the series numbers XI, XII, and XIII, where each pertinent figure represents an average for five animals.

This group of experiments indicates that crowding has a beneficial effect on the rate of tail regeneration.

III. TOXIC SOLUTIONS

A. *Platyopocilus*, in solutions of HgCl_2

Preliminary trials were made to determine the lethal dose of mercuric chloride. As was expected, it was found to be exceedingly toxic. No fishes which were tried lived for thirty days in a concentration above that made by adding one cc. of .001 saturated (9.2×10^{-6} N) HgCl_2 to 3400 cc. of water (i.e., 2.71×10^{-8} N). Some lived for as much as fifteen days, but no more, in slightly stronger concentrations. In the seventeen experiments run, varying weaker concentrations were used, in the attempt to make conditions more favorable for positive growth, for, as will be seen from inspection of table III, series XIV, the majority of the fishes showed a decrease in length.

TABLE III. Series XIV

The effect of heterotypically conditioned water on the growth of *P. rubra* in sublethal solutions of HgCl_2 .
Length of experiment: 30 days.

Fish pair no.	Conditioned water		Control		Saturation of HgCl_2 added	Normality of HgCl_2
	Length at start	Per cent increase	Length at start	Per cent increase		
1	18.8	0.5	19.2	0.0	.001	9.2×10^{-6}
2	19.0	0.0	19.1	0.0	.0005	9.2×10^{-6}
3	17.2	-1.7	17.3	-4.6	.0005	
4	18.0	2.6	18.0	0.0	.0001	4.6×10^{-6}
5	17.9	0.0	19.0	0.0	.0001	
6	18.2	-1.0	18.5	-3.2	.0001	
7	18.5	-2.7	19.6	-4.0	.0001	
8	15.4	-0.6	15.4	-2.6	.000025	2.3×10^{-7}
9	20.2	-1.0	20.5	-2.4	.000025	
10	16.0	-0.6	16.5	-3.0	.000025	
11	15.1	0.0	14.2	0.0	.000001	9.2×10^{-8}
12	10.8	-5.5	10.0	0.0	.000001	
13	10.5	2.7	9.0	0.0	.000001	
14	16.3	4.9	16.5	2.4	.000001	
15	10.0	3.3	9.8	-3.1	.000001	
16	10.7	0.9	9.0 ¹	0.0	.000001	
17	14.0	3.6	14.3	-0.7	.000001	
Mean:	15.7	0.4	15.6	-1.2		
Statistical significance:		.0194				

Of the eight fishes out of the thirty-four which did show an increase in length, six were in tanks having the least amount of poison added.

Two fishes were put in each paraffin lined jar. These jars held 3400 cc. Mussel-conditioned water was aerated and filtered into the experimental jars and unconditioned water was similarly treated for the controls. One cc. of HgCl_2 solution of the desired strength was added at the beginning of the experiments. The water was half changed daily. At the time the change was made, the fishes were removed and put into paraffin lined dishes for feeding. An amount of food was given them slightly in excess of that which they could consume in a half hour. At the end of that time, the fishes were transferred into dishes of clean water, from which they were again removed in a few minutes and replaced in the battery jars. One half cc. of the proper

concentration of HgCl_2 was then added. The jars were washed and the water completely changed weekly. The experiments ran for thirty days.

The average percentage increase for the seven experimental fish showing an increase in length was 2.6 per cent. Only one control fish showed an increase. The table below, given in full to show concentration used in each case, shows that the average percentage increase for all seventeen experimental fishes was 0.4 per cent, and for all the controls, — 1.2 per cent.

B. *Girardinus guppyi* in solutions of NH_4Cl

Experiments on this substance were added, since it occurs in industrial wastes.

The same procedure was followed in these experiments as in those on HgCl_2 . One cc. of .01 concentrated (10 g/L, or .1868 N) NH_4Cl was added at the beginning of the experiment of 3400 cc. of water, and one half cc. replaced daily, with the half changes of water. Ten of the twenty-two fishes (series XV) showed an increase of growth; nine of these ten were in conditioned water. From a mean length at the beginning of the experiment of 12.0 mm. for the fishes in conditioned water and 12.3 mm. for the controls, the former showed a mean increase of 2.9 per cent, and the latter of — 2.2 per cent. This gives a statistical significance of .0030.

These experiments indicate that the presence of an associated animal of a different species may have the effect of favoring growth in media containing sublethal amounts of toxic substances.

CONCLUSIONS

I. In a medium of filtered mussel-conditioned water, as contrasted with non conditioned filtered water, there is:

- A. Greater growth in
 - a. *Platypoecilus rubra*.²
 - b. Larvae of *Ambystoma tigrinum* (uncut tails).
- B. More rapid regeneration of amputated tails in:
 - a. *P. rubra*.
 - b. Larvae of *A. maculatum*.

II. In homotypically conditioned water, as contrasted with non-conditioned water, there is:

- A. As regards growth in groups as contrasted with isolated individuals,
 - 1. Greater increase in the length of uncut tails in *A. tigrinum* in unfiltered, but not in filtered water.
 - 2. Greater decrease in the length of uncut tails in *A. maculatum* in filtered water, when the animals have been in the laboratory several weeks.

² Non-conditioned water unfiltered in experiments shown in series I.

3. No difference, in filtered water, when the available space remains the same, and the available volume is five times as great for the isolated animals as for the groups, and the animals are prevented from coming in contact with each other.
- B. More rapid regeneration of amputated tails in:
 - a. *P. rubra* (conditioned water, not grouped).
 - b. *A. maculatum* in untreated, and also in filtered and aerated water, and *A. tigrinum* in untreated water (grouped).

III. The presence of mussels has a beneficial effect on the growth of fish in water to which a sublethal dose of HgCl_2 or NH_4Cl has been added.

DISCUSSION

We are more familiar, perhaps, with the occurrence of beneficial effects of heterotypic conditioning in nature, than of homotypic conditioning; in fact, the latter has usually been found to produce harmful results, particularly if over-crowding prevails. The phenomena of ecological succession give many supporting considerations for this point of view. In the upland forest succession in sand, the oak associates grow up where the pines have formed sufficient shade and humus, and they, correspondingly, must give way to the beech-maple climax, for they have made changes in the environment unsuitable for themselves, but beneficial for other species which enter as a succeeding stage in ecological succession. The beech-maple forest forms a so-called climax stage in which the forms present no longer produce conditions toxic to themselves. Subject to climatic control such communities exist for long periods, and whatever changes are made in the environment are at least not detrimental to the continued existence of the community.

Such considerations suggest that there are probably many instances where the presence of different species may serve a useful end, to one where the effect of the presence of numbers of the same species is beneficial. Experiences in the course of the present work have tended to support such a difference in emphasis. Clear evidence regarding the beneficial effects of heterotypic conditioning was obtained with a great deal less preliminary exploration than that pertaining to homotypic conditioning. A number of paths of approach, which proved to be blind alleys, were tried, in attempting to find whether there were circumstances under which homotypic conditioning would give positive results. The controlling factors, it seemed, were complex. Preliminary work had mainly to do with various volume relationships. Heterotypic conditioning, on the other hand, was found to have positive effects in much more nearly the first situations tried, which would indicate that the effect was more widespread. It may be that while numbers of the same species may alter the environment in a beneficial direction in a manner quite comparable to that of numbers of a different species, the changes produced in the water by homotypic secretions or excretions or their decom-

position products are more toxic than are those of other species. Thus a nicer balance must be struck, in such matters as volume relationships and frequency of change of medium, in order to realize the possibilities of obtaining positive effects by homotypic conditioning. In this connection there is the work of Woodruff ('14), who found that the excretory products of *Paramecia* are toxic to themselves, and that this is also true of hypotrichs. The excretory products of hypotrichs, however, stimulate the reproductive rate of *Paramecia*, which normally succeed them in protozoan succession.

There was one exception, however, to this general point of view. This occurred in the experiments on the effect of homotypic conditioning on the tail regeneration of tadpoles. In twenty experiments on *A. maculatum*, the chances of random sampling giving so great a difference between experiment and control were practically zero, and it took only seven experiments on *tigrinum* to give a statistical significance of .0010. Besides the fact that these two experiments are among the most clearly significant, statistically, of the entire series reported, the results were obtained after very little preliminary work. They are comparable, therefore, to the type of growth response exhibited to heterotypic conditioning of experimental water. The less elusive evidence for positive effects of homotypic conditioning on regeneration may be due to the fact that the body fluids exuding from the cut surface have a more beneficial effect upon a wounded surface than the secretions or excretions from the normal animal have upon growth. With a number of animals present, the salt and organic content of such an exudate, going into the surrounding medium, would tend to bring the medium into balance with the body fluids of the animals more quickly than they would in the case of the isolated animals.

The growth responses shown by tadpoles to homotypic conditioning are of interest from the standpoint of previous work done in this line. In Bilski's work, from which he concluded that crowding has a deleterious effect, the tadpoles he used had been in the laboratory for a week before he separated them for his experiments, which ran for over a month. Goetsch gives the length of his axolotl larvae as 15 mm. Of the experiments reported above, the set most comparable in age of larvae is that summarized in table II, series VII, which gives results on animals three or four weeks old at the beginning of the seven day experiment in filtered water. As we have seen, these showed a definite negative response to conditioning. Two sets, also in filtered water, showed an indifferent response—one in which network partitions separated the available space from the available volume of water, and prevented collisions between animals, and another without such a network. The conditions under which a definite positive effect of homotypic conditioning was demonstrated included unfiltered water and very young larvae. It seems reasonable to suggest, in the light of these results, that the age of the larvae and the exact composition of the medium may be important factors in determining the effect of homotypic conditioning.

Situations in nature come easily to mind in considering the possible usefulness of a positive effect of heterotypic conditioning. The stream from which the mussels were collected is inhabited by a number of species of small fish. It is quite likely that the presence of the molluscs adds definitely to the favorableness of this stream as a fish habitat, while the well known relation between fishes and pelecypod glochidia makes fish occupancy a necessary prerequisite for these fresh water molluscs. Examples of this sort are easily multiplied. The practice is commonplace of placing snails in aquaria with fish, in order to secure better living conditions for the fish. Quite a parallel suggestion to the above is obvious in reference to experiments involving toxic materials. Although these fresh water mussels are not found where the concentration of industrial wastes is high, there are two possibilities in this connection: first, that the mussels may be positively effective either by conditioning the stream above the point where the wastes are added, or further down stream where the concentration is low enough for them to live; and second, that the presence of other species which can exist in a higher concentration may show the same beneficial effect of heterotypic conditioning as has been found for mussels.

While the positive effects of homotypic conditioning on growth do not seem to be so widespread, they may be equally important where present. The previous work in this laboratory (Allee, '31), and elsewhere, upon the beneficial effects of homotypic crowding in clearing up toxic solutions, so that all or the majority of animals in a group may survive under conditions which would kill isolated animals, indicates that in newly evolved or in polluted environments homotypic conditioning may have significantly beneficial effects.

The absence of harmful effects, which was observed in all experiments except those on older tadpoles, shows that under the conditions of these experiments the animals can tolerate the proximity of other similar animals in the same restricted area. This means that the well known adverse effects of homotypic crowding can be avoided more easily than the beneficial effects can be demonstrated. Such a neutral zone corresponds to the first level of social organization (Allee, '31), the toleration level.

The next question to be answered experimentally concerns the possible mechanism by which the effects of conditioning are produced. There are two broad divisions here; first, is there a removal of toxic material from the well-water used, or, second, is there an addition of a growth promoting substance? In one set only of the above experiments do we find any facts which might bear on this question. Tadpoles in filtered water did not show any significant difference between groups and singles, but those in untreated water did. This suggests that the removal of toxic material by the crowding animals is the effective agent in promoting growth of these tadpoles and that such removal may be accomplished by filtering as well. In connection with the experiments on fish in toxic solutions, the findings of others would indi-

cate that toxic material was being removed; however, no direct tests were made on this point in these experiments.

Further, it is worth pointing out that in all experiments on heterotypic conditioning, and in those on homotypic conditioning in fish, the animals were isolated or in twos, rather than in larger groups. This limits the manner in which the experimental animals could be affected differently from the controls to some change made in the medium, and excludes any mechanism involving direct interaction between the animals, such as is concerned in Bilski's stimulation factor.

Chemical analyses of the water in the Whitman laboratory are in progress, as are also investigations of the protozoan and bacterial content of raw and of conditioned waters. It is to be hoped that these analyses will bring to light just what changes are made in the water by conditioning and will open up opportunities for experimental investigation as to the exact nature of the growth promoting factors in homotypically and heterotypically conditioned media.

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